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Assessing the chemotaxis behavior of *Physarum polycephalum* to a range of simple volatile organic chemicals

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The chemotaxis behavior of the plasmodial stage of the true slime mold *Physarum polycephalum* was assessed when given a binary choice between two volatile organic chemicals (VOCs) placed in its environment. All possible binary combinations were tested between 19 separate VOCs selected due to their prevalence and biological activity in common plant and insect species. The slime mold exhibited positive chemotaxis toward a number of VOCs with the following order of preference:

Farnesene > β-myrcene > tridecane > limonene > p-cymene > 3-octanone > β-pinene > m-cresol > benzylacetate > cis-3-hexenylacetate.

For the remaining compounds, no positive chemotaxis was observed in any of the experiments, and for most compounds there was an inhibitory effect on the growth of the slime mold. By assessing this lack of growth or failure to propagate, it was possible to produce a list of compounds ranked in terms of their inhibitory effect:

nonanal > benzaldehyde > methylbenzoate > linalool > methyl-p-benzoquinone > eugenol > benzyl alcohol > geraniol > 2-phenylethanol.

This analysis shows a distinct preference of the slime mold for non-oxygenated terpene and terpene-like compounds (farnesene, β-myrcene, limonene, p-cymene and β-pinene). In contrast, terpene-based alcohols such as geraniol and linalool were found to have a strong inhibitory effect on the slime mold. Both the aldehydes utilized in this study had the strongest inhibitory effect on the slime mold of all the 19 VOCs tested. Interestingly, 3-octanone, which has a strong association with a "fungal odor," was the only compound with an oxygenated functionality where *Physarum polycephalum* exhibits distinct positive chemotaxis.

Introduction

Physarum polycephalum is a true acellular slime mold that belongs to the species of order *Physarales*, subclass *Myxo-gastromycetidae*, class *Myxomycetes*, division *Myxostelida*. The life cycle of *Physarum polycephalum* possesses a plasmodial phase where it exists as a single cell with a large number of diploid nuclei. The plasmodium is yellow colored and moves like a giant amoeba, deploying a network of protoplasmic tubes while searching for food, which typically consists of bacteria, spores and micro-particles.[1] In the plasmodial stage, the protoplasm is differentiated into relatively stable ectoplasm and fluid endoplasm that streams through ectoplasmic channels and tubular strands. The direction and velocity of endoplasmic flow changes according to the pressure gradient formed via ectoplasm contractions[2] coordinated along the body. This contraction is provided by myosin oligomers interacting with actin filaments attached to the membrane.[3] The period of force autowaves and shuttle streaming of the endoplasm varies in the range of 1–5 min depending on the physiological state of the plasmodium.[4] Any fragment of a plasmodium restores the integrity of the surrounding membrane and resumes the contractile and locomotive activities, therefore, fragments of standard size and shape can be used in chemotactic assays.

Cytoplasm streams rhythmically back and forth through a network of tubular elements, circulating nutrients and chemical signals and forming pseudopods that allow the organism to navigate around and respond to its environment. The plasmodium propagates according to the position of nutrients but also in response to external/environmental gradients in light levels and humidity. It is also well established that Physarum will propagate according to gradients in certain chemical species, either chemoattractants or chemorepellents. The *Physarum polycephalum* plasmodium is a model system for studying non-muscular motility, and its chemotactic behavior has been well documented.[5-8] In particular, substances causing negative taxis (repellents) were shown to increase the period of contractility and to decrease the area of spreading when present uniformly within the substrate.[7,8]

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The first studies on chemoattractants and their effect on Physarum can be traced back over 100 y. There has been constant cyclical interest since the inception of these studies although most studies have focused on nutrient type molecules, or chemicals such as cyclic nucleotides with a known direct biological effect. A good overview of work performed on *Physarum Polycephalum,* which predates the 1960s, can be found in Carlile.[9] Experimental studies confirmed that the following substances acted as chemoattractants for the plasmodium, glucose, galactose, maltose and mannose,[9,10] peptones,[9,11] the amino acids phenylalanine, leucine, serine, asparagine, glycine, alanine, aspartate, glutamate; and threonine,[12-14] phosphates, pyrophosphates, ATP and c AMP and thorium nitrate.[15] A plasmodium is allegedly indifferent to fructose and ribose.[9,10] Whereas, the following compounds have been found to act as chemorepellent molecules, sucrose and inorganic salts such as the chloride salts of (K, Na, NH_4, Ca, Mg, La)[15,16] and tryptophan.[14] Therefore, it is clear that the nutritional value of the substance is not paramount in determining either chemoattractant or chemorepellent properties.[6,13] Although recently there has been renewed interest in the question of nutritional value and chemotaxis.[17] For some substances, the effect on the plasmodium can be determined by the proximity of the organism to the source (or the concentration of the source), meaning that some substances can act as both chemoattractant and chemorepellent molecules. An example is the sugars galactose and mannose, which are reported to act as chemoattractants[9,10] and chemorepellents that inhibit motion.[18]

Physarum polycephalum plasmodium has also been used in chemotactic assays to assess the biocidal and repellent effects of silver nanoparticles vs. silver ions.[19] A number of other metals are known to have a repellent effect on Physarum.[20]

Recently it was found[21] that the plasmodium is strongly attracted to herbal calming/somnipherous tablets Nytol and KalmsSleep. To select the principle chemoattractant in the tablets, laboratory, experiments were undertaken on the plasmodium's binary choice between samples of dried herbs/roots: Valeriana officinalis, Humulus lupulus, Passiflora incarnate, Lactuca virosa, Gentiana lutea and Verbena officinalis. A hierarchy of chemo-attractive force was calculated from the binary interactions and it was found that Valerian root was the strongest chemo-attractant for *P. polycephalum* of the substances tested. Valerian contains hundreds of identified, and possibly the same amount of not-yet-identified components, including alkaloids, volatile oils, valerinol and actinidine. Therefore, it is unclear which component is causing the chemo-attractive effect. However, actinidine is known to have a chemo-attractive effect on a number of other animal species so remains a strong candidate.[22]

Kincaid and Mansour[23] found that inhibitors of the enzyme cyclic 3',5'-AMP phosphodiesterase act as strong or moderate chemoattractants in P. polycephalum. Among the substances tested the strongest effect was observed with 4-(-3-butoxy-4-methoxybenzyl)-2-imidazolidinone and moderate effects from theophylline and other xanthine derivatives (interestingly they observed negative chemotaxis at high concentrations).

In a recent study,[24] it was found that dithiothreitol inhibits the action of cAMP specific phosphodiesterase, which is responsible for the hydrolysis of cAMP. They found that the onset of the plasmodium spreading and the transition to the stage of migration were delayed in a concentration dependent manner. They concluded that the results suggest that the autocrine production of cAMP and extracellular cAMP specific phosphodiesterase is an important constituent of the mechanism controlling the motile behavior of the *Physarum polycephalum* plasmodium.

Recently, the plasmodial phase of *Physarum polycephalum* has been used extensively as a biological computing substrate. It has been used to solve a wide range of computationally hard problems such as maze-solving, the traveling salesman problem, calculation of optimal graphs, construction of logical gates and arithmetic circuits, sub-division of spatial configurations of data points and robot control.[20,25-29] The plasmodium's behavior is determined by external stimuli and excitation waves traveling and interacting inside the plasmodium.[30] The plasmodium can be considered as a reaction-diffusion[31] or an excitable[32] medium encapsulated in an elastic growing membrane.

In Adamatzky,[28] the concept of Physarum machines was introduced. A Physarum machine is a programmable amorphous biological computer experimentally implemented in the plasmodium of *P. polycephalum*. A Physarum machine on a nutrient-rich substrate behaves as an auto-wave in an excitable medium. On a non-nutrient substrate it propagates similarly to a wave fragment in a sub-excitable medium. A Physarum machine can be programmed by configurations of repelling (salt) and attracting (food) gradients, and localized reflectors (illuminated obstacles). Gradient fields generated by discrete configurations of attractants are an important prerequisite for successful programming of Physarum machines.

This paper sets out to investigate the chemoattractant or chemorepellent properties of a range of simple molecules that are mainly identified as secretions from plants and/or insects. By building a database of interactions between Physarum and various chemical species it should be possible to exert fine control over the movement and morphology of the slime mold. As mentioned this is a pre-requisite for the fabrication of successful computing substrates. In addition a fundamental study of the interactions of Physarum with various volatile organic compounds is invaluable in elucidating the underlying metabolic processes.

Results and Discussion

Table 1 shows the results for the 10 replicates of the binary combinations of the 19 VOCs. The result is a quaternary string A-N-FTP-B. (A) indicates the number of replicates in which Physarum propagated toward substance A, (B) indicates the number of replicates in which Physarum propagated toward substance B, (N) indicates the number of neutral propagation events for that binary mixture of chemicals, whereas FTP indicates the number of replicates where Physarum failed to propagate from the inoculation site.

Table 1. Showing the results of a series of binary chemotactic assays utilizing 19 simple VOCs

VOC	1B	2B	3B	4B	5B	6B	7B	8B	9B	10B	11B	12B	13B	14B	15B	16B	17B	18B	19B
1A		0-0-10-0	5-5-0-0	10-0-0-0	7-3-0-0	5-5-0-0	4-6-0-0	8-2-0-0	9-1-0-0	5-5-0-0	0-10-0-0	0-5-0-5	7-3-0-0	1-9-0-0	2-4-0-4	8-2-0-0	3-7-0-0	3-7-0-0	0-0-10-0
2A			0-0-10-0	0-0-10-0	0-0-10-0	0-0-10-0	0-10-0-0	0-0-10-0	0-0-10-0	0-0-10-0	0-0-10-0	0-0-10-0	0-0-10-0	0-0-10-0	0-0-10-0	0-0-10-0	0-0-10-0	0-0-10-0	0-0-10-0
3A				9-1-0-0	7-3-0-0	10-0-0-0	7-3-0-0	8-2-0-0	3-7-0-0	0-0-10-0	0-2-8-0	0-0-10-0	10-0-0-0	10-0-0-0	1-4-0-5	5-4-0-1	4-6-0-0	0-3-0-4	0-0-10-0
4A					1-9-0-0	0-10-0-0	1-9-0-0	0-10-0-0	0-0-10-0	0-0-10-0	0-0-10-0	0-0-0-10	0-7-3-0	0-1-9-0	0-2-0-8	0-7-0-3	0-3-0-7	0-4-0-6	0-0-10-0
5A						0-8-2-0	0-10-0-0	0-2-8-0	0-0-10-0	0-0-10-0	0-0-10-0	0-0-10-0	0-8-2-0	0-0-10-0	0-3-0-7	0-7-0-3	0-4-0-6	0-9-0-1	0-0-10-0
6A							0-2-8-0	0-10-0-0	0-0-10-0	0-0-10-0	0-0-10-0	0-0-10-0	0-10-0-0	0-0-10-0	0-2-0-8	0-8-0-2	0-4-0-6	0-9-0-1	0-0-10-0
7A								0-8-2-0	0-0-10-0	0-0-10-0	0-0-10-0	0-4-0-6	0-9-1-0	0-0-10-0	0-0-0-10	0-6-0-4	0-0-0-10	0-2-0-8	0-0-10-0
8A									3-7-0-0	0-0-10-0	0-0-10-0	0-5-0-5	0-10-0-0	0-0-10-0	0-0-1-9	0-4-0-6	0-2-0-8	0-1-0-9	0-0-10-0
9A										0-0-10-0	0-0-10-0	0-0-10-0	0-0-10-0	0-0-10-0	0-0-9-1	0-10-0-0	0-10-0-0	0-2-7-1	0-0-10-0
10A											0-0-10-0	0-0-10-0	0-0-10-0	0-0-10-0	0-8-1-1	0-10-0-0	0-0-0-10	0-8-2-0	0-0-10-0
11A												0-0-10-0	0-0-10-0	0-0-10-0	0-1-6-3	0-8-2-0	1-9-0-0	0-6-3-1	0-0-10-0
12A													7-3-0-0	0-0-10-0	0-2-0-8	6-2-0-2	2-6-0-2	1-2-7-0	0-0-10-0
13A														0-10-0-0	0-0-0-10	0-6-0-4	0-10-0-0	0-8-0-0	0-0-10-0
14A															0-0-1-9	0-7-0-2	0-4-0-6	0-4-3-3	0-0-10-0
15A																8-2-0-0	4-5-0-1	6-2-0-2	0-0-10-0
16A																	0-10-0-0	3-3-1-3	0-0-10-0
17A																		2-6-0-2	0-0-10-0
18A																			0-0-10-0
19A																			

FORMAT: M[A,B] = [A:N:FTP:B], A = number of experiments (total 10) where Physarum propagates toward A in preference to B; n = number of experiments where Physarum propagates equally toward A and B (They are not chemorepellents, but neither is a strong or dominant chemoattractant), or propagates between A and B (i.e., both A and B have a chemorepellent effect). FTP = number of experiments where Physarum fails to propagate from the initiation site (this is indicative that one or both of the compounds has a strong inhibitory effect on Physarum, by studying the full extent of the binary interactions it becomes apparent as to the individual effect of each VOC) B = number of experiments where Physarum propagates toward B in preference to A; 1 = β-Myrcene 2 = Benzaldehyde 3 = Tridecane 4 = Benzylacetate 5 = Eugenol 6 = Benzylalcohol; 7 = Geraniol 8 = M-Cresol 9 = Linalool 10 = Methylbenzoate 11 = Cis-3-hexenyl acetate 12 = P-cymene;13 = 2-Phenylethanol 14 = Methyl-p-benzoquinone 15 = α-farnesene 16 = β-pinene 17 = limonene 18 = 3-octanone 19 = nonanal

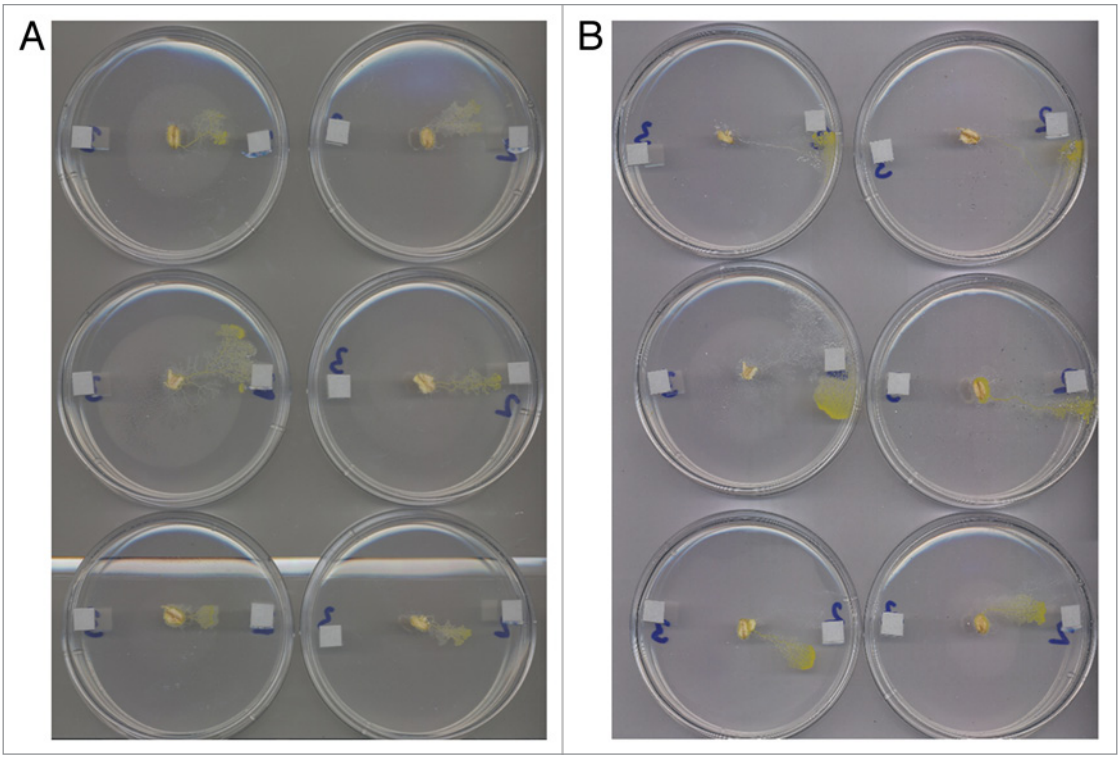

Figure 1. (**A**) Showing selected results from the chemotactic assay between farnesene (right side of the Petri dish) and geraniol (left side of Petri dish) 24 h after initiation. (**B**) The results of the chemotactic assay between farnesene and geraniol after 48 h.

The most common result for a binary mixture of chemicals is for Physarum to fail to propagate from the inoculation site in all 10 replicates (cells marked blue in the table). This occurred 75 times from a total of 171 experiments. This indicates that many of the chemicals have an inhibitory effect on the Physarum either individually or in combination. Compounds that have a predominantly inhibitory effect can be deduced from the binary combinations within the table. These include in order of the greatest inhibitory effect [based on the number of experiments where all 10 replicates gave no propagation (the total number of experiments for each compound was 18)]:

Nonanal (18) > benzaldehyde (17) > methylbenzoate (11) > cis-3-hexenyl acetate (10) > linalool (9) methyl-p-benzoquinone (8) > p-cymene (7) > Eugenol (5) = benzyl alcohol (5) > geraniol (4) > m-cresol (3) = 2-phenylethanol (3) = benzyl acetate (3) > tridecane(2).

Therefore, compounds that show no individual or cumulative inhibitory effect include farnesene, limonene, β-pinene, β-myrcene and 3-octanone.

There were some occasions where Physarum propagated toward either compound A or compound B in all 10 replicates (cells marked red in the table). This indicates that these compounds exert a significant chemoattractive effect. These are relatively rare outcomes, with only 9 instances in 171 total experiments. The compounds observed to have a strong chemoattractive effect were β-myrcene (vs. benzylacetate), tridecane (vs. benzyl alcohol, 2-phenylethanol, methyl-p-benzoquinone), p-cymene (vs. benzyl acetate), farnesene (vs. geraniol and 2-phenylethanol) and limonene (vs. geraniol and methylbenzoate).

There are an additional set of experiments where at least 5 of the 10 replicates indicated a positive chemotactic response (cells marked green in the table). The compounds with the highest number of these events were as follows:

farnesene (9) > β-myrcene (8) > tridecane (6) > p-cymene = limonene (5) > 3-octanone (3) > β-pinene (1)

There were a number of experiments (14/171) where all 10 replicates resulted in a neutral outcome, indicating that neither chemical had a strong inhibitory effect or a strong chemoattractive effect (cells marked yellow in the table). In addition, there were a number of experiments (40/171) where the overall result was neutral. This is where most of the 10 replicates gave either a neutral outcome or failed to propagate, and only a limited number of replicates indicated a positive chemotactic response.

We can also rank the compounds in terms of their total number of positive chemotactic events when looking at all replicates in all experiments. This gives the following ranking: (the number in brackets indicates the number of positive events out of a total of 180).

farnesene (101) > β-myrcene (77) > tridecane (74) > limonene (58) > p-cymene (42) > 3-octanone (41) > β-pinene (30) > m-cresol (3) > benzyl acetate (2) > cis-3-hexenyl acetate (1).

The other 9 compounds gave no positive chemotactic events in all the replicate experiments. From these results, and the ones above, it is obvious that farnesene has the strongest chemoattractive effect on *Physarum polycephalum*, followed by myrcene

and tridecane, which have a relatively strong effect and then limonene/p-cymene/3-octanone and β-pinene that have a moderate effect.

If we rank the remaining compounds in terms of their total number of inhibitory events then we get the following order

nonanal (180) > benzaldehyde (170) > methyl benzoate (130) > linalool (126) > methyl-p-benzoquinone (113) > Eugenol (88) > benzyl alcohol (72) > geraniol (69) > 2-phenylethanol (67).

This shows that both aldehydes studied have the greatest inhibitory effect with Physarum failing to propagate in all replicates vs. all other compounds regardless of their chemoattractive properties. The list of inhibitory substances is also populated by all the alcohols utilized in the study, although the effect is much less marked than that observed for aldehydes.

Figure 1A shows selected results from the chemotactic assay between farnesene (right side of the Petri dish) and geraniol (left side of the Petri dish) 24 h after initiation. In all cases, there is a definite positive chemotaxis toward farnesene. The Physarum takes a direct horizontal path from the source of initiation toward the farnesene. The growth rates within each dish are different, but eventually all reactions proceed to the same point whereby the Physarum encircles the source of farnesene. In some cases (as shown in the dish on the middle row, left side), the Physarum colonizes the filter paper substrate that had been soaked in farnesene. This seems to show that there is no inhibitory effect even at close proximity to the source. **Figure 1B** shows the results of the chemotactic assay between farnesene and geraniol after 48 h. What is interesting is that Physarum remains in the localized area surrounding the source of farnesene, despite there being no additional nutrient source. Often Physarum may move initially toward a source, but then subsequently move away in search of nutrients.

Figure 2 shows selected results of the chemotactic assay between farnesene (right side) and pinene (left side) after 48 h. Again, it is clear that there is a positive chemotactic response to farnesene. However, in contrast to **Figure 1A** (where geraniol was the other source), the growth is not as localized and does not take a direct horizontal path toward farnesene. Instead, where pinene is the alternative source, then Physarum takes a more circuitous route toward the source of the farnesene. This probably indicates that pinene does not have a direct inhibitory effect on the growth of Physarum. However, as seen with the case of geraniol, there is very limited growth on the entire side of the Petri dish not containing farnesene.

Figure 3 shows selected results from the chemotactic assay using limonene (right side) and 2-phenyl ethanol (left side). In this case, no chemotaxis is observed toward either of the chemical compounds. In fact, the majority of growth is on a vertical line from the source of initiation. Growth is also limited to a localized region around the source of initiation. Therefore, at least one of the compounds appears to have an inhibitory effect on the Physarum, and neither has a strong chemoattractive action.

Figure 4 shows selected results from the chemotactic assay between limonene (right side) and 3-octanone (left side). The growth of the Physarum is relatively unhindered and it is able to grow out from the point of initiation in all directions. In terms of an overall result, there is no chemotaxis toward one source in

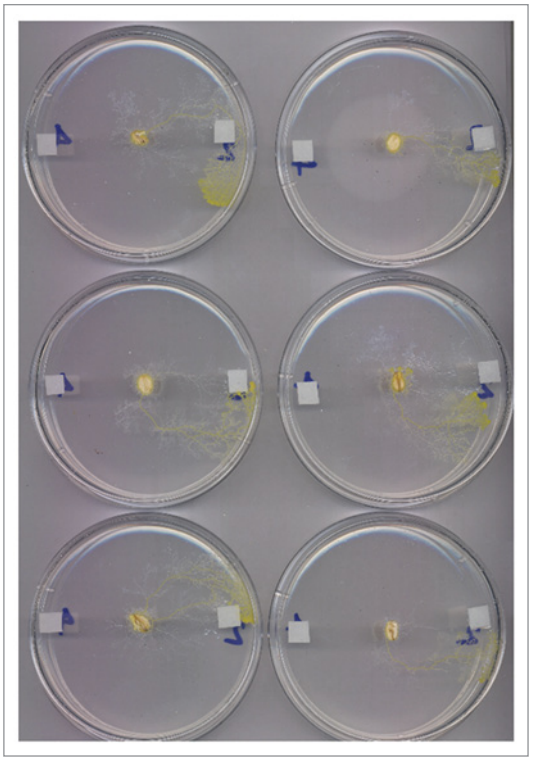

Figure 2. Selected results of the chemotactic assay between farnesene (right side) and pinene (left side) after 48 h.

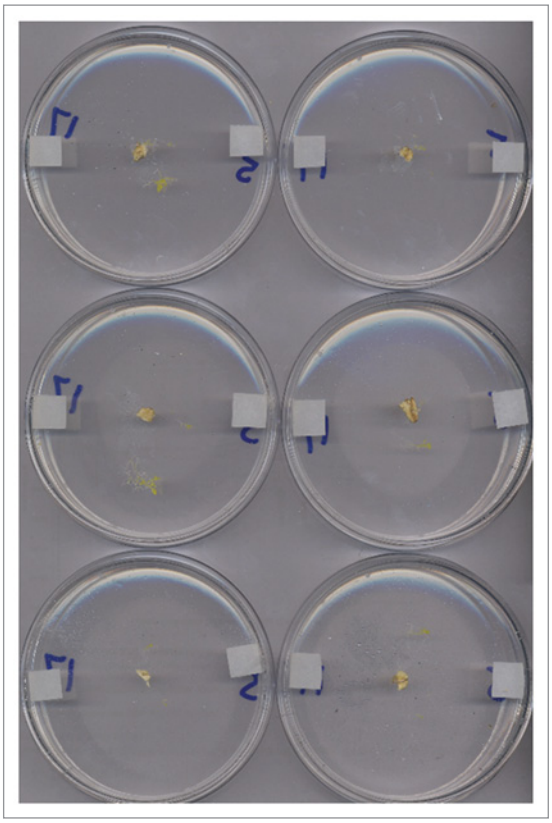

Figure 3. Showing selected results from the chemotactic assay using limonene (right side) and 2-phenyl ethanol (left side).

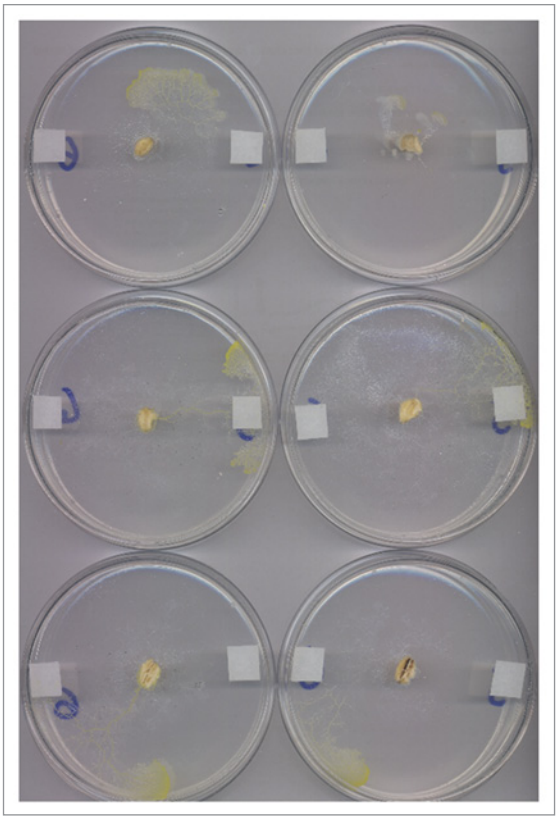

Figure 4. Showing selected results from the chemotactic assay between limonene (right side) and 3-octanone (left side).

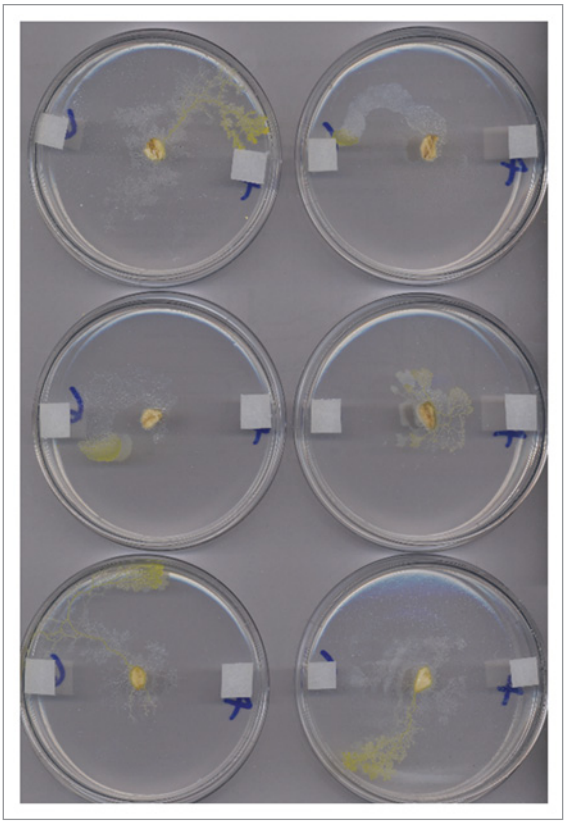

Figure 5. Showing selected results from the chemotactic assay between pinene (right side) and 3-octanone (left side).

preference to another. Therefore, it can be concluded that neither of the chemical substances has a strong inhibitory effect on Physarum, and both appear to have a moderate chemoattractive action.

Figure 5 shows selected results from the chemotactic assay between pinene (right side) and 3-octanone (left side). Again, it is obvious that neither compound has a strong inhibitory affect on the Physarum. The growth is not directly toward the source of either chemical. In some cases, the growth is in all directions, as in the example on the right side, middle row. In other cases, it appears to grow at a 45-degree angle to the source and then follow an almost circular trajectory, eventually reaching the source (this occurs for both chemicals; see for example top left side, moving toward pinene, whereas top left side and middle row, left side, moving toward 3-octanone). Actually, the movement toward 3-octanone is interesting because it takes the form of a discrete wave fragment. This wave fragment follows the same trajectory in both cases.

Figure 6 shows selected results from the chemotactic assay between 3-octanone (right side) and benzyl acetate (left side). There is an obvious chemotaxis toward 3-octanone in preference to benzyl acetate. What is interesting is that the Growth of the Physarum is limited to compact fragments and many of these take different trajectories from the source and move in the general direction of 3-octanone. There is very little growth in the half of the Petri dish occupied by the source of benzyl acetate. Therefore, it seems likely that the growth of the Physarum is altered by the long range inhibitory effect of benzyl acetate in its environment. This shows how the morphology and growth rate of Physarum can be altered by its chemical environment. This wave fragment like morphology is qualitatively similar to fragments of excitation which can exist in the sub-excitable BZ reaction.[33] Previously, these fragments of excitation have been utilized in collision-based computing schemes to implement ballistic gates. In this case, wave fragments were controlled by subtle gradients in the applied illumination field. This allowed fine control over the trajectory and velocity vectors of the wave fragments, and for the classification of binary collisions to be undertaken. Indeed, the interaction of localized waves of *Physarum Polycephalum* have been previously used for implementing arithmetic circuits.[34] However, in this case, the Physarum is confined to certain channels in order to implement computation. It is plausible that finely balanced inhibition coupled with positive chemotaxis could be used to exert fine control over the evolution and movement of these fragments enabling collision-based computing schemes to be explored. This would be in an architectureless domain, albeit it pervaded by chemical fields. It is also possible that this morphology would be useful for material transport and directed synthesis of functional materials.

In the controls where a single substance was present at both sources, the predominant behavior was neutral. Where the chemical was a strong attractant (farnesene, β myrcene) then Physarum

propagated toward both sources although not necessarily to both in one replicate. Where the chemical was a strong inhibitor then the Physarum failed to propagate. Physarum did not exhibit any chemotactic response toward distilled water. Where distilled water alone was used as the two sources the propagation was predominantly neutral.

The above analysis shows definite trends in terms of the types of compounds that possess inhibitory and chemoattractive responses to *Physarum polycephalum*. In general terms oxygen functionality seems to exhibit an inhibitory affect on the propagation. In particular aldehydes seem to have the strongest inhibitory affect. All the alcohols seem to populate the inhibitory group although with a lesser inhibitory effect observed than with aldehydes. Most of the aromatic compounds tested also populate the inhibitory group. In terms of chemoattractive properties all the terpene derivatives without oxygen functionality populate this group with the only sesquiterpene farnesene exhibiting the highest overall effect. The fatty acid derivative tridecane also has a high chemoattractive effect while 3-octanone is unique in being the only oxygenated compound to show even a moderate chemoattractive affect.

All the terpene molecules exerting a chemoattractive response share certain structural features such as the lack of oxygen functionality, unsaturation etc. However, there are distinct differences, farnesene and β-myrcene are straight chain, acyclic terpenes, limonene is cyclic and pinene is bicyclic, whereas p-cymene is classed as a terpene like compound but is aromatic. These molecules do not obviously show any similarity to previously identified compounds exhibiting a strong chemoattractive response to Physarum. The role of phosphodiesterase inhibitors as chemoattractants for Physarum has been previously studied.[23] Many of these compounds were cyclic and contained nitrogen functionality such as xanthine derivatives. These compounds are non-selective phosphodiesterase inhibitors meaning that they raise intracellular cAMP. A recent paper has described a key role of cAMP and extracellular cAMP phosphodiesterase in the motile behavior of *P. polycephalum*.[24] Limonene and other terpenes have been found to bind to A(2A) adenosine receptors in humans.[35] Other antagonists are caffeine, theophylline, istradefylline. In addition, limonene increased cytosolic cAMP concentration and calcium concentration, which can be achieved by the activation of adenosine A(2A) receptors. Physarum is known to have a calcium sensitive contractile system.[36] Therefore, it is possible that these terpenes act directly to increase motility in Physarum via enzyme inhibition. It is well documented that the hydrophobicity of certain terpene molecules contributes to their effectiveness as enzyme inhibitors.[37,38] Many hydrophobic compounds are associated with protein or enzyme deactivation, where acetylcholinesterase is particularly sensitive. Compounds that inhibit or inactivate acetylcholinesterase cause acetylcholine to accumulate at synapses of cholinergic sites. This produces continuous stimulation of cholinergic fibers at neuromuscular junctions. Several essential oil monoterpenes demonstrated a competitive inhibition of acetylcholinesterase. Specific acetylcholinesterase has previously been identified in the plasmodial stage of Physarum suggesting that it has a role as a local mediator of motor function.[39,40]

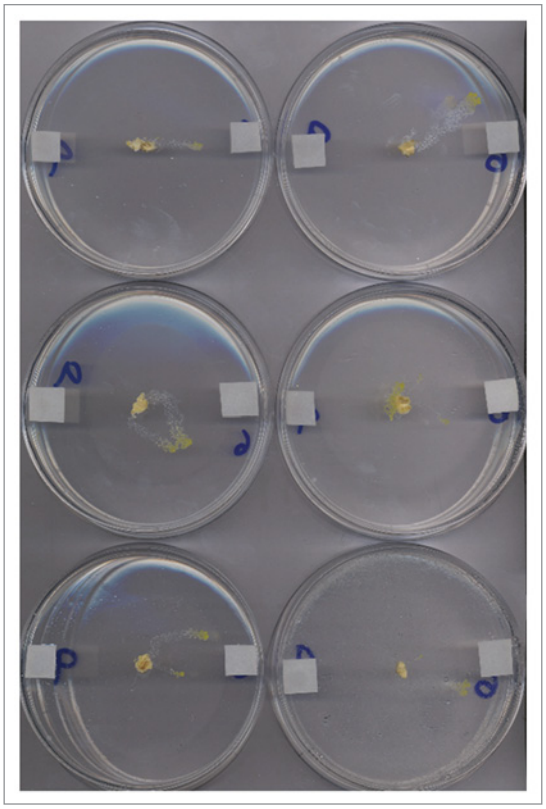

Figure 6. Selected results from the chemotactic assay between 3-octanone (right side) and benzyl acetate (left side).

3-octanone is one of the compounds that contributes to a characteristic fungal odor, which Physarum possesses, although the most obvious odor emanating from Physarum is that of geosmin. GC-MS analysis was recently undertaken[41] of the headspace above living Physarum and identified geosmin as one of the major volatile components. Geosmin has a very low odor threshold in humans and has a characteristic earthy flavor. It is produced by a number of bacterial species, including actinobacteria especially Streptomyces. The biosynthesis was found to be via farnesyl diphosphate.[42] In fact, many organisms use this pre-cursor in the production of terpenes, terpenoids and sterols.

A number of terpene based compounds were observed in the headspace above Physarum including "farnesene like" compounds (as there are very many terpene like molecules with similar mass and fragmentation patterns it is necessary to run standards in order to gain a positive identification) 3-octanone and other "typical fungal volatiles."

We intend to use the compounds identified in the GC-MS study to augment these experiments in terms of assessing their chemoattractive or chemorepellent effect. This study has established a fairly robust preference list ranked in terms of attraction and repulsion and therefore, it should be relatively facile to establish the position of new compounds such as those identified as secretions of Physarum via GCMS within the exisiting order. In the future experiments will be performed to ascertain any physiological effects on the slime mold by making electrical measurements of Physarum with and without various chemoattractive

and inhibitory compounds in the environment. Using this methodology we can also control the concentration of the VOCs and gauge any dose response effects or whether certain compounds exhibit both chemoattractive and inhibitory responses at different concentration levels. In addition to a fundamental study of the volatile secretions of Physarum and the effect of relatively simple VOCs on the propagation of the plasmodium, the aim is to use the information gained through this study and extended studies in order to exert fine control over the propagation direction/speed and the morphology of the slime mold.

This work identified a number of previously unknown compounds that exert chemotactic response from the plasmodial stage of *P. Polycephalum*. A number of relatively simple VOCs were found to have either a chemoattractive or chemorepellent action when tested in binary chemotactic assays with *Physarum Polycephalum*. The compound exhibiting the highest chemotactic response was farnesene. A number of other terpenes or terpene like molecules without oxygen functionality (β-myrcene, limonene, β-pinene and p-cymene) were found to exert a moderate chemoattractive response. Whereas terpene compounds with oxygen functionality such as geraniol and linalool exhibited a moderate inhibitory effect. Aldehydes were found to exhibit the greatest inhibitory effect on the growth/propagation of the plasmodium. Alcohols and aromatic compounds also had a predominantly inhibitory effect.

Previously chemoattractive response has been linked to the inhibition of phosphodiesterase enzymes. More recently a role for c-AMP and c-AMP phosphodiesterase enzymes in the motile behavior of *P. Polycephalum* has been established. We can postulate based on associated evidence that the terpene like molecules (especially those with high hydrophobicity) have an inhibitory effect on enzymes linked to the motile response of *Physarum polycephalum* thus causing a direct chemotactic effect.

This work also highlighted the potential for altering the morphology of the plasmodium in response to chemical stimuli. In the presence of moderate inhibitory compounds for example it was possible to reduce the typical extended morphology of the plasmodium to compact wave fragments.

It is hoped that a better understanding of the chemotaxis response of *Physarum Polycephalum* to simple VOCs could provide a basis for fine control of the propagation direction, morphology and growth rate. This would be invaluable in giving a programmable substrate for applications in computation, amorphous robotics and material synthesis. From a fundamental perspective it is also invaluable to understand the semiochemicals of slime molds and their complex relationships with other organisms in their environment such as plant and insect species. The chemotaxis response of primitive eukaryotic cells is also useful as a model for pathogenesis.

Materials and Methods

Selection of compounds for use in chemotactic assays. A recent paper by Schiestl[43] detailed the evolution of floral scent and insect chemical communication. This involved an analysis of the occurrence, commonness and evolutionary patterns of 71 "floral" VOCs in 96 plant families and 87 insect families. We used this paper as a basis for selecting 16 compounds. The compounds were selected from each of the four chemical classes studied in the paper namely aromatic, monoterpenes, sesquiterpenes and fatty acid derivatives (FADs). We aimed to get a range of different functionalities within these generic groups and also select compounds which had been identified across a high number of plant and insect families. The compounds selected were as follows: 6 aromatics (benzaldehyde, benzyl alcohol, methyl benzoate, benzylacetate, 2-phenyl ethanol and Eugenol), 6 monoterpenes (Limonene, β-myrcene, Geraniol, Linalool, p-Cymene and β-pinene), a sesquiterpene (α-farnesene) and 3 FADs [(Z)-3-Hexenyl acetate, nonanal and tridecane]. These compounds were all purchased from Sigma Aldrich UK and used as received.

In addition to these 16 compounds, we also selected 3-octanone a known fungal metabolite[44] and m-cresol and methyl-p-benzoquinone, which are known secretions of the fungus beetle *Bolitotherus cornutus* and a range of other insect species,[45]

Culturing of *Physarum polycephalum*. The true slime mold, the plasmodium of *Physarum polycephalum* (strain HU554 × HU560), was cultured with oat flakes on a 1% agar gel at 25°C in the dark. To obtain large quantities of inoculated oat flakes for chemotactic assays the plasmodial phase of *P. polycephalum* was cultivated in large plastic containers on kitchen towels that have been wetted with 5 mls of de-ionized water. Any excess water was removed from the container. A source of food was added in the form of 50 g of rolled oats per container (Organic rolled oats). These containers were covered in order to retain moisture and kept in the dark at 25°C until required. These cultures were checked daily and water added if required. Sub-cultures were taken every 2–3 d to establish consistent cultures for ongoing experiments. Sub-culturing simply involved the removal of colonized oat flakes from the main culture and addition to a separate container containing fresh rolled oats on damp kitchen paper.

Chemotactic assays. We use a scheme similar to that adopted by other researchers undertaking simple chemotactic assays.[19,21] Experiments were performed in 9 cm diameter polystyrene Petri dishes. A 1% solution of agar (Select Agar) was added to each Petri dish to give a depth of approximately 2 mm. Squares of filter paper (*ca.* 0.5 cm^3) were cut and placed on the gel surface at the furthest points from the center on a straight line. An oat flake colonized by *Physarum polycephalum* was placed at the center of the Petri dish, on a straight line connecting the two filter paper substrates and at the same distance from each substrate. The plasmodium was left on the Petri dish in the dark for two hours prior to the addition of the chemicals. After two hours, the dishes were removed to a fume cupboard and 50 μL of the selected chemicals were added to the filter paper substrates (in the case of methyl-p-benzoquinone, 50 mg of solid was added).

Ten replicates were set up for each set of binary chemical assays. The Petri dishes were then sealed and kept in the dark. They were checked at six hourly intervals for evidence of any chemotactic effect. Usually between 24–48 h, the chemotactic behavior was established and recorded. All dishes were scanned in batches of six were taken using a flatbed scanner (HP Scanjet

5590) attached to a PC. The assessment of the behavior in each dish was according to four classifications. If the plasmodium propagated from the inoculation site directly (approximately horizontally) to a certain chemical source, then this was counted as a positive chemotactic event (it could obviously equally indicate a strong negative chemotactic effect away from the other source). The results for each chemical source were added for each of the 10 Petri dishes in order to establish whether a statistically significant trend existed for one source vs. the other. Therefore, a series of preferences could be established by testing all the possible binary combinations of the 19 reactants.

If *Physarum* propagated horizontally but equally toward both sources, or simply propagated in a circular or random direction then this was classified as a balanced chemotactic response. In this case the Physarum had no clear preference for either chemical source but the growth was not significantly inhibited. Again, the number of this type of event from the 10 replicates of the chemotactic assay were summed to give an overall indication of the "typical behavior."

If *Physarum* failed to propagate from the site of inoculation, then this was highly indicative of a direct inhibitory effect of one or both chemical sources. The number of these events was noted and summed over the replicates for that binary assay.

A number of control experiments were undertaken including selected trials run with distilled water replacing one or both chemicals. We also ran a limited subset of experiments where the same compound was present at both sources on the Petri dish.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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